ORIGINAL ARTICLE 10.1111/j.1469-0691.2007.01768.x

Characterisation of human outbreaks of brucellosis and sporadic cases by the use of hyper-variable octameric oligonucleotide fingerprint (HOOF) variable number tandem repeats

S. Valdezate[1,2], I. Cervera[1], P. Hernandez[3], A. Navarro[1] and J. A. Saéz Nieto[1]

[1]Departmento de Bacteriología, [2]Unidad de Alertas y Emergencias, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid and [3]Servicio de Microbiología, Hospital de San Pedro Alcántara, Cacéres, Spain

ABSTRACT

Hyper-variable octameric oligonucleotide fingerprints (HOOFs) enable typing of *Brucella* spp. by targeting the 8-bp tandem repeat in eight loci that vary in number (variable number tandem repeats; VNTRs). *Brucella* is one of the most important zoonotic pathogens, because of its public health and economic consequences. To assess the role of HOOFs as epidemiological markers for *Brucella melitensis*, which is the main species involved in human brucellosis in Spain, 87 sporadic and outbreak isolates were investigated; these originated from broad or more restricted geographical locations, including unrelated (n = 42), semi-related (n = 19) and closely related (n = 26) groups of isolates. Distinct HOOFs were detected in the entire (n = 74), unrelated (n = 42), semi-related (n = 19) and closely related (n = 13) groups. Seven of the eight VNTR markers investigated identified multiple alleles in the four groups of isolates. Using the composite data for eight VNTRs, a diversity value of 0.98 was calculated for the entire population, taking into account single- and double-locus variants. A high correlation (R = 0.98) between the maximum copy number and the number of alleles was observed. The most polymorphic markers were VNTR-1, VNTR-4, VNTR-5 and VNTR-7 ($D \geq 0.8$). Characterisation of *B. melitensis* isolates by HOOFs enabled the recognition of related human cases and the exchange of molecular epidemiological information concerning a spreading clone, thus improving brucellosis surveillance.

Keywords *Brucella melitensis*, epidemiology, HOOFs, outbreaks, typing, variable number tandem repeats

Original Submission: 24 November 2006; **Revised Submission:** 7 March 2007; **Accepted:** 14 April 2007

Clin Microbiol Infect 2007; **13**: 887–892

INTRODUCTION

Brucella spp. are major zoonotic pathogens responsible for a chronic debilitating disease in humans, with non-specific systemic influenza-like symptoms that localise in multiple organ systems and cause severe manifestations, including osteoarticular disease, hepatitis and endocarditis [1–3]. The most frequent routes of acquisition for humans involve the consumption of unpasteurised contaminated dairy products or the inhalation of infected aerosol particles [3,4]. Brucellosis is considered to be an occupational disease for workers in contact with farm animals and for laboratory personnel. This organism has also been implicated as a possible agent of bioterrorism [5–8].

Brucella causes considerable human morbidity in endemic areas worldwide, as well as economic losses in animal husbandry. Spain, in common with other Mediterranean countries, is not officially brucellosis-free [2,7], but the incidence in humans has declined steadily during the last 4 years [9,10]. The incidence of brucellosis was 1.5 cases/100 000 inhabitants in 2004 (595 human cases). Major foci, involving 31.1%, 18.3% and

Corresponding author and reprint requests: S. Valdezate, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Crta. Majadahonda-Pozuelo Km2, Madrid 28220, Spain
E-mail: svaldezate@isciii.es

14.7% of brucellosis cases (185, 109 and 88 cases, respectively), were located in Andalucía, Extremadura and Castilla La Mancha autonomous region [10]. However, the true incidence of brucellosis could be even higher, as subjects with active brucellosis do not always show symptoms of illness [11].

The high homogeneity revealed in DNA–DNA hybridisation experiments, as well as in the 16S rRNA gene, has led to the suggestion that the genus *Brucella* comprises a single species, *Brucella melitensis* [12,13], although this proposal remains controversial and requires further investigation using large-scale sequencing studies [14–15]. However, the apparent lack of genetic polymorphism [16,17] has restricted the development of typing methods for epidemiological surveillance of brucellosis [18]. Fortunately, recent findings have provided new opportunities for molecular typing [19–21], and the hyper-variable octameric oligonucleotide fingerprint (HOOF) technique [22] is a promising tool for characterising *Brucella* isolates.

In order to study human cases of the disease and to investigate the possible foodborne or occupational sources of infection, the present study investigated the use of HOOFs based on variable number tandem repeats (VNTRs) [23] as molecular markers for *B. melitensis* isolates responsible for human cases of brucellosis in Spain. To date, this typing method has been widely applied to study isolates of *Brucella abortus* and *B. melitensis* [24,25]. Sporadic and outbreak strains collected from human cases of brucellosis in Spain during a 9-year period were analysed. The isolates tested were classified into three groups on the basis of different degrees of geographical linkage.

MATERIALS AND METHODS

Bacterial strains

Eighty-seven *B. melitensis* isolates from humans, submitted to the Bacteriology Department of the National Center of Microbiology (CNM), Madrid, Spain, were investigated. These comprised three groups of isolates: (i) a geographically unrelated group comprising 42 isolates from 16 Spanish provinces during 1997–2005; (ii) a geographically semi-related group comprising 19 isolates from the province of Córdoba, Andalucía, during 1999–2001; and (iii) a geographically closely related group comprising 26 isolates collected mainly from a more restricted geographical area surrounding rural villages in Coria, in the province of Caceres (Extremadura), during 2004–2005. Only one isolate per patient was included in the study. *B. abortus* B19, *B. melitensis* REV1 and *B. melitensis* 16M, as well as *Brucella suis*, *Brucella ovis* and *Brucella canis* strains from the CNM collection, were included as control strains. Species identification by PCR, based on the different localisation of the IS*711* genetic element in the *Brucella* chromosome, yielded a 731-bp amplicon for strains of *B. melitensis* [26].

VNTR-PCR and sequencing of amplified products

PCR assays were performed as described by Bricker *et al*. [22], using primers without fluorescent dyes for amplification and sequencing. DNA was obtained by resuspension of bacteria in 1 mL of distilled water, heat inactivation at 100°C for 20 min, and subsequent centrifugation to remove cellular debris. Amplification was performed using PureTaq Ready-To-Go PCR Beads (Amersham Biosciences, Chalfont St Giles, UK) with 0.25 μM each primer. PCR conditions comprised 94°C for 2 min, followed by 32 cycles of 94°C for 15 s, 55°C for 20 s and 72°C for 90 s, with a final extension at 72°C for 5 min. PCR products (5 μL) were analysed by electrophoresis in MS-8 agarose (Pronadisa, Conda, Madrid, Spain) 3% w/v gels at 90 V for 1 h. A 50-bp DNA ladder (Amersham Biosciences) was used to provide molecular size standards.

The number of repeats of the sequence AGGGCAGT, located at eight loci in the genome, was determined by sequencing and by generating strain-specific fingerprints. PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). DNA sequencing was performed using the ABI Prism Big Dye Terminator Kit in conjunction with an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical and phylogenetic analyses

Alleles were defined at each VNTR locus according to the number of repeat units found, and a HOOF type (HT) was assigned to each isolate, based on the combination of the alleles for VNTRs 1–8. The discriminatory power of each locus was estimated by the number and range of allele frequencies (maximum − minimum number of repeats), and by the diversity index, $D = 1 - \sum(\text{allele frequency})$ [27], for each of the three geographical groups, as well as for all 87 isolates studied. Clustering of the HTs obtained was performed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and UPGMA (unweighted pair-group method using arithmetic averages) with the categorical option (which considers character states to be unordered).

RESULTS

VNTR marker diversity

Each *B. melitensis* isolate yielded a range of amplified fragments, with sizes of 50–200 bp. In order to analyse VNTR variability, the number of alleles, and the range, mode and diversity index were determined for seven of the eight loci for each group, and also for the entire population (Table 1). VNTR-3 was not considered in the analysis, because it was monomorphic for *B. melitensis*. VNTR-1, VNTR-4, VNTR-5 and VNTR-7 showed

Table 1. Hyper-variable octameric oligonucleotide fingerprint (HOOF) variable number tandem repeat (VNTR) characteristics among human *Brucella melitensis* isolates, including single-locus variants (SLVs) and double-locus variants (DLVs) for each locus and geographical group

Group/ VNTRs[a]	Unrelated (*n* = 42)	Semi-related (*n* = 19)	Closely related (*n* = 26)	Total (*n* = 87)
VNTR-1				
No. of alleles	10, 2 DLV	8	4, 1 SLV	10
Range	2–12	3–12	4–9	2–12
Mode	8	7	9	9
D	0.80	0.83	0.33	0.84
VNTR-2				
No. of alleles	5, 1 DLV	5	5	5
Range	2–6	2–6	2–6	2–6
Mode	4	4	4	4
D	0.62	0.66	0.28	0.57
VNTR-4				
No. of alleles	11	8, 1 SLV	4, 1 SLV, 1 DLV	14
Range	1–14	3–17	3–15	1–17
Mode	6	8	6	6
D	0.85	0.76	0.45	0.75
VNTR-5				
No. of alleles	14, 1 DLV	8, 1 SLV	6	14
Range	2–16	4–13	4–12	2–16
Mode	8	6/7	6	6
D	0.89	0.82	0.47	0.81
VNTR-6				
No. of alleles	7	4	5	7
Range	1–10	2–6	1–6	1–10
Mode	4	6	5	5
D	0.76	0.62	0.29	0.81
VNTR-7				
No. of alleles	13	9, 1 SLV	4, 1 DLV	13
Range	3–15	3–12	4–7	3–15
Mode	3/6/8	11	4	4
D	0.89	0.84	0.28	0.86
VNTR-8				
No. of alleles	5	5	4, 2 SLV	5
Range	2–6	2–6	2–5	2–6
Mode	2	2	3	2
D	0.48	0.71	0.37	0.54

D, marker diversity.
[a]VNTR-3 was not analysed, as it showed an absence of polymorphism in *B. melitensis*.

the highest number of alleles in the different groups (Fig. 1). Allele distributions for VNTR-5 and VNTR-7 are shown in Fig. 2. A high correlation (*R*) value of 0.98 was found between the

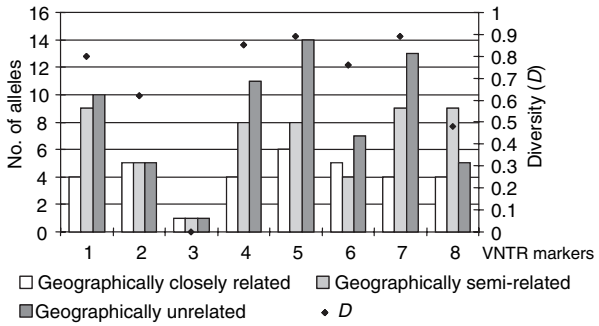

Fig. 1. Number of alleles and allelic diversity detected at eight *Brucella melitensis* variable number tandem repeats (VNTR) loci. Filled diamonds indicate Nei's diversity index (*D*) analysed across 42 hyper-variable octameric oligonucleotide fingerprint (HOOF) types corresponding to the geographically unrelated group.

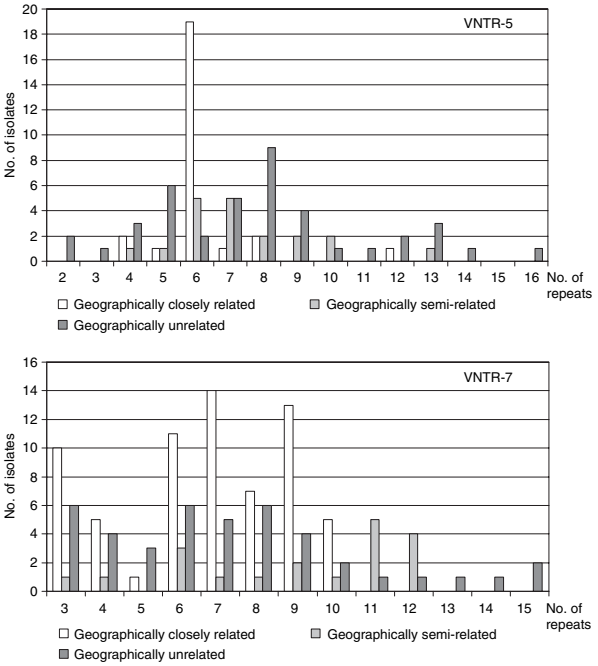

Fig. 2. Allele distributions (number of repeats) of the variable number tandem repeat (VNTR) loci with the highest diversity, i.e., VNTR-5 (upper) and VNTR-7 (lower).

maximum copy number and the number of alleles observed in the unrelated group (*n* = 42) and in the entire population (*n* = 87) for the eight VNTRs considered using the HOOF technique. When individual HTs were considered for each isolate in the entire population, the diversity reached a value of 0.987 for the composite data from the eight VNTRs, while the typeability was equal to 1.

Genetic relationships among isolates based on VNTR analysis

When UPGMA clustering was used to assess the genetic relationships among the 74 different HTs obtained for 87 isolates from humans, 42, 19 and 13 HTs were identified in the geographically unrelated, semi-related and closely related groups, respectively. Four genogroups (A, B, C and D) and three subgroups (B1–B3) were defined, based on similarities of <25% and ≥30%, respectively, with similarity scores ranging from 22% to 100% (Fig. S1; see Supplementary material). Several *B. melitensis* human isolates with similar HTs (≤2 alleles different among the eight VNTRs) were grouped in genogroups A, B1, B2 and C. These single-locus variants (SLVs) and

double-locus variants (DLVs) represented 33.3%, 40.7%, 87.5% and 10% of these genogroups, respectively. Among the SLVs and DLVs, two were geographically unrelated isolates, ten were semi-related isolates, and 21 were closely related isolates. *B. melitensis* 16M and *B. melitensis* REV1 were both located in major genogroup B (Fig. S1), while *B. abortus* B19 and the CNM control strains of *B. suis*, *B. ovis* and *B. canis* were located outside the above genogroups (data not shown).

Use of HOOFs for discriminating outbreak isolates from sporadic cases

In the geographically unrelated group, the 42 isolates displayed 40 different HTs with ≥three differences in the number of repeat copies; two isolates recovered from two patients from the province of Huelva were DLVs with VNTR-1,2 variations.

In the geographically semi-related group, 12 isolates showed unique HTs, while seven isolates displayed similar HTs; of these, five were SLVs with changes in VNTR-4 or VNTR-7, and two were DLVs with changes in VNTR-7 and VNTR-8.

In the geographically closely related *B. melitensis* group, similarity within HTs was increased, with a high genetic similarity coefficient (≥90%). Similar HTs were identified among isolates collected from 20 patients (Fig. S1). The main profiles within these HTs were: HT-o1 (9,4,1,6,6,5,4,3) in ten isolates; HT-o2 (9,4,1,6,6,5,4,2) in four isolates; and HT-o3 (9,4,1,7,6,5,4,2) in two isolates. Four isolates displayed other HTs with at least two differences in comparison with HT-o1: (i) a decrease of five repeat units, VNTR-1(−5u); (ii) an increase of one repeat unit, VNTR-8 (+1); (iii) VNTR-7(+1u), 8(−1u); and (iv) VNTR-5(−1u) and VNTR-3(−2u). Investigation of the clinical charts of the patients from whom isolates with these highly similar HTs were obtained revealed that all were involved in a single outbreak in Coria (Cacéres), in which brucellosis was acquired following the consumption of goat cheese.

DISCUSSION

Brucellosis remains one of the most important zoonoses in Spain [9,10]. This is also the case in other EU and Mediterranean countries, in the USA, and in Central and South American countries [5,11,28–30]. Between 2000 and 2004, 4197 cases of human brucellosis were notified in different Spanish regions, with the identification of 109 outbreaks [10]. The special efforts made to decrease animal and human *Brucella* infections resulted in a 52% decrease in human cases in 2004 compared with 2000. However, several foci of the disease are still apparent in Andalucía, Extremadura and Castilla La Mancha autonomous communities [9,10,31], which are areas that have a high number of husbandry farms. The main reasons favouring persistence of an endemic brucellosis situation in many countries include the uncontrolled movement of animals, an absence of veterinary advice, contact with infected farm animals, ingestion of raw milk derivatives, and international travel [4,20].

Unlike the USA, where *B. abortus* is mainly responsible for cases of human brucellosis [29], *B. melitensis* is responsible for 97.5% of notified cases of human infection in Spain [10] and other EU and Mediterranean countries [32,33]. This has been linked to the presence of an infected ovicaprine population [10,28,34] in areas with a high incidence of human brucellosis [28,35]. The primary objectives of human brucellosis surveillance are to identify new human infections and to determine whether infections are of foodborne or occupational origin [20].

Molecular characterisation of microorganisms based on the analysis of tandem repeat sequences has revealed extensive length polymorphisms, even in bacteria that are thought to be genetically homogeneous [36–38]. HOOFs have been shown to be capable of differentiating *Brucella* isolates by the identification of variability in the 8-bp tandem repeat [22]. Multilocus characterisation using PCR has been demonstrated to discriminate between isolates of *B. abortus* [22,24] and *B. melitensis* [22,25]. The present study reduced the manipulation of *Brucella* isolates to a minimum by using a boiling method to extract DNA, and amplicons were resolved by conventional agarose gel electrophoresis rather than capillary electrophoresis. This is the first time that HOOFs have been used to analyse a large collection of human *B. melitensis* isolates with different epidemiological characteristics. Using seven VNTR loci, the diversity coefficient for HOOFs reached a high value of 0.98; this slightly decreased to 0.95 when SLV and DLV HTs were considered to belong to the same clone.

The loci with the highest diversity index (≥0.8) were VNTR-5, VNTR-7, VNTR-4 and VNTR-1,

both in the geographically unrelated group and in the entire population. If the efficacy of a specific VNTR locus is linked to the number of repetitive copies, VNTR-5, VNTR-7, VNTR-4 and VNTR-1 are effective tools for studying isolates from a single country, as they yielded the maximum number of alleles (16–12 repeats). Therefore, in specific situations, amplification and sequencing could be reduced to VNTR-1, VNTR-4, VNTR-5 and VNTR-7 in order to obtain preliminary typing results.

In the present study, isolates in the geographically unrelated group were allocated to all phylogenetic genogroups, the geographically semi-related isolates were not found in genogroup C, and 80.7% of the closely related isolates collected in a geographically restricted area were allocated to cluster B2 (Fig. 1). Accordingly, HTs with at least two differences (similarity range 65–100%) can be considered to belong to the same clone. Most cases of brucellosis in the geographically unrelated group were sporadic, with the identification of 40 HTs. The only exception involved two patients from Madrid in 2003 whose isolates were DLVs, with changes in VNTRs 2(−1u) and 5(−1u). The cases in the geographically semi-related group included ten sporadic cases and nine cases that could be linked within four outbreaks involving occupational acquisition. An outbreak was also suspected for the isolates from 20 patients, grouped in the geographically closely related group, that displayed similar HTs (Fig. S1). The first profile detected was HT-o1 (9, 4, 1, 6, 6, 5, 4, 3), which was identified in isolates from ten patients during a 42-day period. During the same period, isolates from ten other patients showed six similar HTs (SLVs, DLVs), with one or two changes in VNTR-1, VNTR-3, VNTR-4, VNTR-5, VNTR-7 and VNTR-8, compared with HT-o1. The source of brucellosis was linked to the consumption of unpasteurised cheese.

Based on the findings for the semi-related and closely related groups, VNTRs used in the HOOF technique appear to offer a promising approach for the characterisation of *B. melitensis* isolates, thereby enabling the detection of related cases among humans and the identification of the source of infection, i.e., foodborne or occupational. New VNTRs have recently been described for identification and typing [38,39], and may provide new opportunities for surveillance of animal and human brucellosis. However, not all VNTRs are equally effective for characterising *B. melitensis*, with three of 13 VNTRs having a diversity index ≥0.80, while HOOFs authenticated six of seven VNTRs [38].

In conclusion, the greater diversity and higher mutation rates of the VNTRs used in this study allow high-resolution analysis of epidemics, regardless of the level of the epidemiological link. The recognition, management and control of an epidemic/outbreak of human brucellosis can be improved by the use of a safe, rapid and reproducible typing technique, such as HOOF analysis. The numerical profiles assigned to each clone can be exchanged among laboratories in order to characterise and trace the strains involved in outbreaks. Targeting of the octameric sequence in seven of the eight loci of *B. melitensis* defined by HOOFs should enable the improvement of brucellosis surveillance and control programmes focused on safeguarding public health.

ACKNOWLEDGEMENTS

We thank the clinical microbiologists, particularly A. M. Blázquez and F. De Carlos, involved in the isolation and submission of *Brucella* isolates to the Taxonomy Laboratory at CNM. We thank M. A. Usera for critical review and M. Harper for revising the English language of the manuscript. I. Cervera and A. Navarro were supported by technical grants from the Comunidad de Madrid and the Instituto de Salud Carlos III, respectively.

SUPPLEMENTARY MATERIAL

The following supplementary material for this article is available online at http://www.blackwell-synergy.com:

Fig. S1. Dendogram showing the UPGMA clustering for 74 hyper-variable octameric oligonucleotide fingerprint (HOOF) types (HTs).

REFERENCES

1. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis* 1997; **3**: 213–221.
2. Pappas G, Akrtidis N, Bosilkovski M, Tsianos E, Brucellosis. *N Engl J Med* 2005; **352**: 2325–2336.
3. Young EJ. An overview of human brucellosis. *Clin Infect Dis* 1995; **21**: 283–289.
4. Mendez Martínez C, Paez A, Cortes-Blanco M *et al.* Brucellosis outbreak due to unpasteurized raw goat cheese in Andalucia (Spain). *Euro Surveill* 2003; **8**: 164–168.
5. Bouza E, Sanchez-Carrillo C, Hernan Gomez S *et al.* Laboratory-acquired brucellosis: a Spanish national survey. *J Hosp Infect* 2005; **61**: 80–83.

6. De Massi F, Di Girolamo A, Petrini A, Pizzigallo E, Giovannini A. Correlation between animal and human brucellosis in Italy during the period 1997–2002. *Clin Microbiol Infect* 2005; **11**: 632–636.
7. Godfroid J, Kashborer A. Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Vet Microbiol* 2002; **90**: 135–145.
8. Yagupsky G, Baron EJ. Laboratory exposures to brucellae and implications for bioterrorism. *Emerg Infect Dis* 2005; **11**: 1180–1185.
9. Martínez F, Sánchez LP, Larrosa A. Estudio epidemiológico de la brucelosis eu España. In: *Manual de brucelosis de la Junta de Castilla y León*, 2001; **63**: 77 (http://www.jcyl.es).
10. Sanchéz-Serrano LP, Ordoñez P, Díaz MO, Torres A. Vigilancia de la brueclosis. *Bol Epidemiol Inst Salud Carlos III* 2004; **12**: 209–220.
11. Wallach JC, Samartino LE, Efron A, Baldi PC. Human infection by *Brucella melitensis*: an outbreak attributed to contact with infected goats. *FEMS Immunol Med Microbiol* 1997; **19**: 315–321.
12. Da Costa M, Guillou JP, Garin-Bastuji B, Thiébaud M, Dubray G. Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. *J Appl Bacteriol* 1996; **81**: 267–275.
13. Verger J, Grimont F, Pad G, Grayon M. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* 1985; **35**: 292–295.
14. Cutler SJ, Whatmore AM, Commander NJ. Brucellosis, new aspects of an old disease. *J Appl Microbiol* 2005; **98**: 1270–1281.
15. Del Vecchio VG, Kapatral V, Redkar RJ *et al*. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc Natl Acad Sci USA* 2002; **99**: 443–448.
16. Halling SM, Peterson-Burch DB, Bricker BJ *et al*. Completion of the genome sequence of *Brucella abortus* and comparison with the highly similar genomes of *Brucella melitensis* and *Brucella suis*. *J Bacteriol* 2005; **187**: 2715–2726.
17. Gandara B, Merino AL, Rogel MA, Martínez Romero E. Limited genetic diversity of *Brucella* spp. *J Clin Microbiol* 2001; **39**: 235–240.
18. Moreno E, Cloeckaert A, Moriyón I. *Brucella* evolution and taxonomy. *Vet Microbiol* 2002; **90**: 209–227.
19. Cloeckaert A, Grayon M, Grepinet O. Identification of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the *rpsL* gene. *Vaccine* 2002; **20**: 2546–2550.
20. Robinson A. Guidelines for coordinated human and brucellosis surveillance. *EMPRES* 2003; **4**: 11–15.
21. Whatmore AM, Murphy TJ, Shankester S, Young E, Cutler SJ, Macmillan AP. Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *J Clin Microbiol* 2005; **43**: 761–769.
22. Bricker B, Ewalt DR, Halling S. *Brucella* HOOF-prints: strain typing by multi-locus analysis of variable number repeats (VNTRs). *BMC Microbiol* 2003; **3**: 15.
23. Weir BS. *Genetic data analysis*. Sunderland, MA: Sinauer Associates, 1990.
24. Bricker B, Halling SM. Evaluation of the HOOF-print assay for typing *Brucella abortus* strains isolated from cattle in the United States: results with four performance criteria. *BMC Microbiol* 2005; **5**: 37.
25. Whatmore AM, Shakster SJ, Perrett LL *et al*. Identification and characterization of variable number-repeat markers for typing of *Brucella* spp. *J Clin Microbiol* 2006; **44**: 1982–1993.
26. Bricker B, Halling SM. Differentiation of *Brucella abortus* bv.1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv.1 by PCR. *J Clin Microbiol* 1994; **32**: 2660–2666.
27. Hunter PR, Gaston MA. Numerical index of the discrimination ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 1988; **26**: 2465–2466.
28. Dokuzoguz B, Ergonul O, Baykam N *et al*. Characteristics of *B. melitensis* versus *B. abortus* bacteraemias. *J Infect* 2005; **50**: 41–45.
29. Troy SB, Rickman LS, Davis CE. Brucellosis in San Diego: epidemiology and species-related differences in acute clinical presentations. *Medicine (Baltimore)* 2005; **84**: 174–187.
30. Luna-Martínez JE, Mejia-Teran C. Brucellosis in Mexico: current status and trends. *Vet Microbiol* 2002; **90**: 19–30.
31. Rodríguez Torres A, Abad R, Orduña R. Especies y biovars del género *Brucella*. Etiología de la brucelosis humana en España. *Enferm Infecc Microbiol Clin* 1992; **10**: 43–48.
32. Al Dahouk S, Nockler K, Hensel A *et al*. Human brucellosis in a nonendemic country: a report from Germany, 2002 and 2003. *Eur J Clin Microbiol Infect Dis* 2005; **24**: 450–456.
33. Avdikou J, Maipa V, Alamanos Y. Epidemiology of human brucellosis in a defined area of northwestern Greece. *Epidemiol Infect* 2005; **133**: 905–910.
34. Caporale V, Nannini D, Giovannini A, Morelli D, Rasmasco M. Prophylaxis and control of brucellosis due to *Brucella melitensis* in Italy: acquired and expected results. In: *Prevention of brucellosis in the Mediterranean countries. Proceedings of the international seminar CIHEAM CEC, MINACG (Malta), FIS (Malta)*. Valletta: CIHEAM, 1992; 127–145.
35. Davis CE, Troy SB. Brucellosis. *N Engl J Med* 2005; **352**: 1071–1072.
36. Lindstedt BA. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis* 2005; **26**: 2567–2582.
37. Van Belkum A, Scherer S, Van Alphen L, Verbrugh H. Short-sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev* 1998; **62**: 275–293.
38. Yazdankhah SP, Lindstedt BA, Caugant DA. Use of variable-number tandem repeats to examine genetic diversity of *Neisseria meningitis*. *J Clin Microbiol* 2005; **43**: 1699–1705.
39. Le Fleche P, Jacques I, Grayon M *et al*. Evaluation and selection of tandem repeat assay for a *Brucella* MLVA typing assay. *BMC Microbiol* 2006; **6**: 9.